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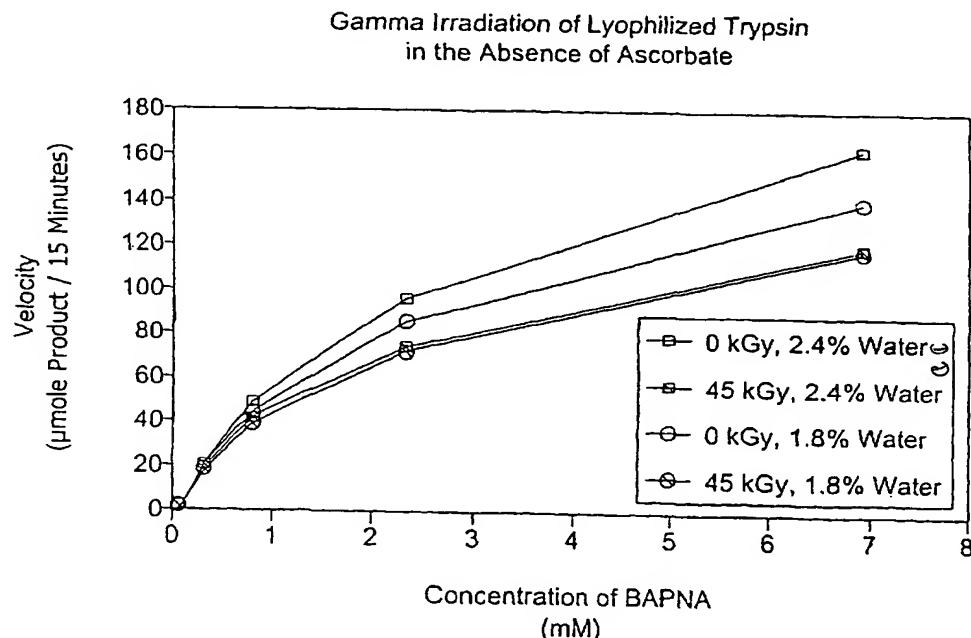
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[Continued on next page]

(54) Title: METHODS FOR STERILIZING PREPARATIONS OF DIGESTIVE ENZYME



(57) Abstract: Methods are disclosed for sterilizing preparations of digestive enzymes to reduce the level of one or more active biological contaminants or pathogens therein, such as viruses, bacteria (including inter- and intracellular bacteria, such as mycoplasmas, ureaplasmas, nanobacteria, chlamydia, rickettsias), yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites. These methods involve sterilizing preparations of digestive enzymes, such as trypsin, α -galactosidase and iduronate-2-sulfatase, with irradiation.

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Methods for Sterilizing Preparations of Digestive Enzymes

Field of the Invention

The present invention relates to methods for sterilizing preparations of digestive enzymes to reduce the level of one or more active biological contaminants or pathogens therein, such as viruses, bacteria (including inter- and intracellular bacteria, such as mycoplasmas, ureaplasmas, nanobacteria, chlamydia, rickettsias), yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites. The present invention particularly relates to methods of sterilizing preparations of digestive enzymes, such as trypsin, α -galactosidase and iduronate 2-sulfatase, with irradiation.

Background of the Invention

The principal foods upon which an organism, such as a human, survives can be broadly categorized as carbohydrates, fats and proteins. These substances, however, are useless as nutrients without the process of digestion to break down foods.

Digestion of carbohydrates begins in the mouth and stomach. Saliva contains the enzyme ptyalin (an alpha-amylase), which hydrolyses starch into maltose and other small polymers of glucose. The pancreatic alpha-amylase is similar to the salivary ptyalin, but several times as powerful. Therefore, soon after chyme empties into the duodenum and mixes with pancreatic juice, virtually all of the starches are converted into disaccharides and small glucose polymers. These disaccharides and small glucose polymers are hydrolysed into monosaccharides by intestinal epithelial enzymes.

Digestion of proteins begins in the stomach. The enzyme pepsin, which is produced in the stomach, digests collagen, a major constituent of the intercellular connective tissue of meats. This enzymatic reaction is essential so that other digestive

involved in the uptake, breakdown, transport, storage, release, metabolism and catabolism of nutrients into forms required and useable by the cell(s) of an organism at various places and times. This includes storage of lipids and their metabolism into energy sources as well as their catabolism and synthesis into other useful compounds.

5 Digestion may also occur as a part of an organism's normal process(es) of tissue generation and regeneration or repair of degraded, damaged or abnormal tissue(s) or molecules. It may also be a feature of or result from apoptosis, immune reactions, infections, neoplasms and other abnormal or disease states of an organism.

10 Preparations of digestive enzymes are therefore often provided therapeutically to humans and animals.

For example, in cases of pancreatitis and lack of pancreatic secretion, preparations of certain pancreatic enzymes, including combinations of lipase, protease and amylase (such as Creon™, Cotazym™, Donnazyme™, Ku-Zyme™ HP, Pancrease™ and Pancrease™ MT, Ultrase™ and Ultrase™ MT, Viokase™, and Zymase™) and combinations of lipase, protease, amylase and cellulase (such as Ku-Zyme™ and Kutrerase™), are administered to ensure proper patient nutrition. The digestive enzymes of particular interest, for example in replacement therapy in humans and animals, therefore include pancreatic digestive enzymes, such as trypsin and chymotrypsin, and functional mutants, variants and derivatives thereof.

20 Trypsin is an enzyme that acts to degrade protein; it is often referred to as a digestive enzyme, or proteinase. In the digestive process, trypsin acts with the other proteinases to break down dietary protein molecules to their component peptides and amino acids. Trypsin continues the process of digestion (begun in the stomach) in the small intestine where a slightly alkaline environment (about pH 8) promotes its maximal enzymatic activity. Trypsin, produced in an inactive form by the pancreas, is remarkably similar in chemical composition and in structure to the other chief pancreatic proteinase, chymotrypsin. Both enzymes also appear to have similar mechanisms of action; residues of histidine and serine are found in the active sites of both. The chief difference between

Gaucher's Disease is a somewhat-similar genetically transmitted disorder, in which harmful quantities of another fatty substance, glucocerebroside, accumulate in the spleen, liver, lungs, bone marrow and brain. Patients suffer from a deficiency in β -glucocerebrosidase, which catalyzes the first step in the biodegradation of glucocerebroside, which arises from the biodegradation of old red and white blood cells. Clinical manifestations include enlargement of the spleen and liver, low blood platelets, fatigue and, in certain forms, progressive brain damage. Enzyme replacement therapy by infusion of a preparation of a modified form of glucocerebrosidase, known as algucerase (CeredaseTM) has been tested and found to be a promising potential therapy for this condition (Barton, *et al.*, "Replacement Therapy for Enzyme Deficiency: Macrophage-targeted Glucocerebrosidase for Gaucher's Disease." *New Engl. J. Med.*, May 23, 1991.).

Mucopolysaccharidoses are a group of inherited metabolic disorders caused by a deficiency in the lysosomal enzymes needed to break down mucopolysaccharides, long chains of sugar molecules used to build connective tissue and organs in the body. A deficiency in one or more of these enzymes causes a build up of excess amount in the body, causing progressive damage and eventual death. Among these disorders are Hurler, Scheie and Hurler/Scheie syndromes (the most severe form, occurs in infancy with death resulting before age 10 years, symptoms include clouding of the cornea and progressive physical and mental disability, caused by a deficiency in α -L-iduronidase), Hunter syndrome (affects juveniles with death usually resulting by age 15 years, symptoms include joint stiffness, mental deterioration, dwarfing and progressive deafness, caused by a deficiency in iduronate-2-sulfatase), Sanfillipo syndrome (death usually occurs by late teens, symptoms include progressive dementia and mental deterioration in childhood, caused by a deficiency in heparan N-sulfatase, α -N-acetylglucosaminadase, acetyl-CoA-glucosaminide acetyltransferase and/or N-acetylglucosamine-6-sulfatase), Morquio syndrome (appears in infancy, symptoms include severe dwarfing and corneal clouding, cardiac or respiratory disease may cause death in third or fourth decade of life, caused by a deficiency in galactosamine-6-sulfatase and/or β -galactosidase), Maroteauz-Lamy syndrome (resembles Hurler

Preparations of digestive enzymes that are prepared for human, veterinary, diagnostic and/or experimental use may contain unwanted and potentially dangerous biological contaminants or pathogens, such as viruses, bacteria (including inter- and intracellular bacteria, such as mycoplasmas, ureaplasmas, nanobacteria, chlamydia, rickettsias), yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites. Consequently, it is of utmost importance that any biological contaminant in the preparation be inactivated before the product is used. This is especially critical when the preparation is to be administered directly to a patient, for example in human therapy corrected or treated by intravenous, intramuscular or other forms of injection. This is also critical for the various preparations that are prepared in media or via culture of cells or recombinant cells which contain various types of plasma and/or plasma derivatives or other biological materials or are used to prepare biological materials for human use and which may be subject to mycoplasma, prion, bacterial, viral and/or other biological contaminants or pathogens.

Most procedures for producing preparations of digestive enzymes have involved methods that screen or test the preparation for one or more particular biological contaminants or pathogens rather than removal or inactivation of the contaminant(s) and/or pathogen(s) from the preparation. Preparations that test positive for a biological contaminant or pathogen are merely not used. Examples of screening procedures include the testing for a particular virus in human blood from blood donors. Such procedures, however, are not always reliable and are not able to detect the presence of certain viruses, particularly in very low numbers, and in the case of as yet unknown viruses or other contaminants or pathogens that may be in blood. This reduces the value or certainty of the test in view of the consequences associated with a false negative result. False negative results can be life threatening in certain cases, for example in the case of Acquired Immune Deficiency Syndrome (AIDS). Furthermore, in some instances it can take weeks, if not months, to determine whether or not the preparation is contaminated. Therefore, it would be desirable to apply techniques that would kill or inactivate

Heat treatment requires that the product be heated to approximately 60°C for about 70 hours which can be damaging to sensitive products. In some instances, heat inactivation can actually destroy 50% or more of the biological activity of the product.

5 Filtration involves filtering the product in order to physically remove contaminants. Unfortunately, this method may also remove products that have a high molecular weight. Further, in certain cases, small viruses and similarly sized contaminants and pathogens, such as prions, may not be removed by the filter.

10 The procedure of chemical sensitization involves the addition of noxious agents which bind to the DNA/RNA of the virus and which are activated either by UV or other radiation. This radiation produces reactive intermediates and/or free radicals which bind to the DNA/RNA of the virus, break the chemical bonds in the backbone of the DNA/RNA, and/or cross-link or complex it in such a way that the virus can no longer replicate. This procedure requires that unbound sensitizer is washed from products since the sensitizers are toxic, if not mutagenic or carcinogenic, and cannot be administered to 15 a patient.

Irradiating a product with gamma radiation is another method of sterilizing a product. Gamma radiation is effective in destroying viruses and bacteria when given in high total doses (Keathly *et al.*, "Is There Life After Irradiation? Part 2," *BioPharm* July-August, 1993, and Leitman, USe of Blood Cell Irradiation in the Prevention of Post 20 Transfusion Graft-vs-Host Disease," *Transfusion Science* 10:219-239 (1989)). The published literature in this area, however, teaches that gamma radiation can be damaging to radiation sensitive products, such as blood, blood products, enzymes, protein and protein-containing products. In particular, it has been shown that high radiation doses are injurious to red cells, platelets and granulocytes (Leitman). U.S. Patent No. 4,620,908 discloses that protein products must be frozen prior to irradiation in order to maintain the 25 viability of the protein product. This patent concludes that "[i]f the gamma irradiation were applied while the protein material was at, for example, ambient temperature, the material would be also completely destroyed, that is the activity of the material would be

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) adding to a preparation of one or more digestive enzymes at least one stabilizer in an amount effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the material.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) reducing the residual solvent content of a preparation of one or more digestive enzymes to a level effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) reducing the temperature of a preparation of one or more digestive enzymes to a level effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes.

Another embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) applying to the preparation of one or more digestive enzymes a stabilizing process selected from the group consisting of: (a) reducing the residual solvent content of a preparation of one or more digestive enzymes, (b) adding to the preparation of one or more digestive enzymes at least one stabilizer, and (c) reducing the temperature of the preparation of one or more digestive enzymes; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to

The invention also provides a biological composition comprising at least one preparation of one or more digestive enzymes wherein the total protein concentration of the preparation is effective to preserve the preparation of one or more digestive enzymes for its intended use following sterilization with radiation.

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Brief Description of the Drawings

Figures 1A-1B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer and at varying levels of residual solvent content.

10 Figure 2 is a graph showing the activity of liquid or lyophilized trypsin following gamma irradiation in the presence of a stabilizer and at varying pH levels.

Figures 3A-3B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer.

15 Figures 4A-4B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer and at varying levels of residual solvent content.

Figures 5A-5B are graphs showing the activity of lyophilized trypsin following gamma irradiation in the absence or presence of a stabilizer and at varying levels of residual solvent content.

20 Figure 6 is a graph showing the activity of trypsin suspended in polypropylene glycol following gamma irradiation at varying levels of residual solvent content.

Figure 7 is a graph showing the activity of trypsin following gamma irradiation in an aqueous solution at varying concentrations of stabilizers.

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any living organism, or involved in the metabolism, catabolism, storage and mobilization of externally or internally-derived nutrients or the breakdown products of tissue and/or cellular repair, regeneration, or removal, such as the following: pancreatic enzymes, including pancreatic proteolytic enzymes, such as trypsin and chymotrypsin, pancreatic lipase and pancreatic amylase; salivary enzymes, such as ptyalin; intestinal enzymes, including intestinal polypeptidases, intestinal amylases and intestinal lipases; glycosidases, such as α -galactosidase; and sulfatases, such as iduronodate-2-sulfatase.

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As used herein, the term "sterilize" is intended to mean a reduction in the level of at least one active biological contaminant or pathogen found in the preparation being treated according to the present invention.

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As used herein, the term "biological contaminant or pathogen" is intended to mean a contaminant or pathogen that, upon direct or indirect contact with a preparation of one or more digestive enzymes, may have a deleterious effect on the digestive enzymes or upon a recipient thereof. Such biological contaminants or pathogens include the various viruses, bacteria (including inter- and intracellular bacteria, such as mycoplasmas, ureaplasmas, nanobacteria, chlamydia, rickettsias), yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and/or single or multicellular parasites known to those of skill in the art to generally be found in or infect preparations of digestive enzymes. Examples of biological contaminants or pathogens include, but are not limited to, the following: viruses, such as human immunodeficiency viruses and other retroviruses, herpes viruses, filoviruses, circoviruses, paramyxoviruses, cytomegaloviruses, hepatitis viruses (including hepatitis A, B and C), pox viruses, toga viruses, Epstein-Barr viruses and parvoviruses; bacteria, such as *Escherichia*, *Bacillus*, *Campylobacter*, *Streptococcus* and *Staphylococcus*; nanobacteria; parasites, such as *Trypanosoma* and malarial parasites, including *Plasmodium* species; yeasts; molds; mycoplasmas and ureaplasmas; chlamydia; rickettsias, such as *Coxiella burnetti*; and prions and similar agents responsible, alone or in combination, for one or more of the disease states known as transmissible spongiform encephalopathies (TSEs) in mammals,

including 6,8-dimercapto-octanoic acid (lipoic acid) and its derivatives and analogues (alpha, beta, dihydro, bisnor and tetranor lipoic acid), thioctic acid, 6,8-dimercapto-octanoic acid, dihydrolopoate (DL-6,8-dithioloctanoic acid methyl ester), lipoamide, bisnor methyl ester and tetranor-dihydrolipoic acid, furan fatty acids, oleic and linoleic and palmitic acids and their salts and derivatives; flavonoids, phenylpropanoids, and flavenols, such as quercetin, rutin and its derivatives, apigenin, aminoflavone, catechin, hesperidin and, naringin; carotenes, including beta-carotene; Co-Q10; xanthophylls; polyhydric alcohols, such as glycerol, mannitol; sugars, such as xylose, glucose, ribose, mannose, fructose and trehalose; amino acids and derivatives thereof, such as histidine, N-acetylcysteine (NAC), glutamic acid, tryptophan, sodium caprylate, N-acetyl tryptophan and methionine; azides, such as sodium azide; enzymes, such as Superoxide Dismutase (SOD) and Catalase; uric acid and its derivatives, such as 1,3-dimethyluric acid and dimethylthiourea; allopurinol; thiols, such as glutathione and reduced glutathione and cysteine; trace elements, such as selenium; vitamins, such as vitamin A, vitamin C (including its derivatives and salts such as sodium ascorbate and palmitoyl ascorbic acid) and vitamin E (and its derivatives and salts such as tocopherol acetate and alpha-tocotrienol); chromanol-alpha-C6; 6-hydroxy-2,5,7,8-tetramethylchroma-2 carboxylic acid (Trolox) and derivatives; extraneous proteins, such as gelatin and albumin; tris-3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186); citiolone; puerarin; chrysins; dimethyl sulfoxide (DMSO); piperazine diethanesulfonic acid (PIPES); imidazole; methoxypsonalen (MOPS); 1,2-dithiane-4,5-diol; reducing substances, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT); cholesterol; probucol; indole derivatives; thimerosal; lazazoid and tirlazad mesylate; proanthenols; proanthocyanidins; ammonium sulfate; Pegorgotein (PEG-SOD); N-*tert*-butyl-alpha-phenylnitro (PNB); 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol); mixtures of ascorbate, urate and Trolox C (Asc/urate/Trolox C); proteins and peptides, such as glycylglycine and carnosine, in which each amino acid may be in its D or L form; diosmin; pupurogalin; gallic acid and its derivatives including but not limited to propyl gallate, sodium formaldehyde sulfoxylate and silymarin. Particularly preferred examples

porphorins; halogenated or metal atom-substituted derivatives of dihematoporphyrin esters, hematoporphyrin derivatives, benzoporphyrin derivatives, hydrodibenzoporphyrin dimaleimide, hydrodibenzoporphyrin, dicyano disulfone, tetracarbethoxy hydrodibenzoporphyrin, and tetracarbethoxy hydrodibenzoporphyrin dipropionamide; doxorubicin and daunomycin, which may be modified with halogens or metal atoms; netropsin; BD peptide, S2 peptide; S-303 (ALE compound); dyes, such as hypericin, methylene blue, eosin, fluoresceins (and their derivatives), flavins, merocyanine 540; photoactive compounds, such as bergapten; and SE peptide.

As used herein, the term "radiation" is intended to mean radiation of sufficient energy to sterilize at least some component of the irradiated preparation of one or more digestive enzymes. Types of radiation include, but are not limited to, the following: (i) corpuscular (streams of subatomic particles such as neutrons, electrons, and/or protons); and (ii) electromagnetic (originating in a varying electromagnetic field, such as radio waves, visible (both mono and polychromatic) and invisible light, infrared, ultraviolet radiation, x-radiation, and gamma rays and mixtures thereof). Such radiation is often described as either ionizing (capable of producing ions in irradiated materials) radiation, such as gamma rays, and non-ionizing radiation, such as visible light. The sources of such radiation may vary and, in general, the selection of a specific source of radiation is not critical provided that sufficient radiation is given in an appropriate time and at an appropriate rate to effect sterilization. In practice, gamma radiation is usually produced by isotopes of Cobalt or Cesium, while X-rays are produced by machines that emit X-radiation, and electrons are often used to sterilize materials in a method known as "E-beam" irradiation that involves their production via a machine.

As used herein, the term "to protect" is intended to mean to reduce any damage to the preparation of one or more digestive enzymes being irradiated, that would otherwise result from the irradiation of that material, to a level that is insufficient to preclude the safe and effective use of the material following irradiation. In other words, a substance or process "protects" a preparation of one or more digestive enzymes from radiation if the

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Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) reducing the temperature of a preparation of one or more digestive enzymes to a level effective to protect the preparation of one or more digestive enzymes from radiation; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes.

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Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) applying to the preparation of one or more digestive enzymes a stabilizing process selected from the group consisting of: (a) reducing the residual solvent content of a preparation of one or more digestive enzymes, (b) adding to the preparation of one or more digestive enzymes at least one stabilizer, and (c) reducing the temperature of the preparation of one or more digestive enzymes; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes, wherein the stabilizing process and the rate of irradiation are together effective to protect the preparation of one or more digestive enzymes from radiation.

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Another preferred embodiment of the present invention is directed to a method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation comprising: (i) applying to the preparation of one or more digestive enzymes at least two stabilizing processes selected from the group consisting of: (a) reducing the residual solvent content of a preparation of one or more digestive enzymes, (b) adding to the preparation of one or more digestive enzymes at least one stabilizer, and (c) reducing the temperature of the preparation of one or more digestive enzymes; and (ii) irradiating the preparation of one or more digestive enzymes with radiation at an effective rate for a time effective to sterilize the preparation of one or more digestive enzymes, wherein the

typically less than about 8%, usually less than about 5%, preferably less than about 3.0%, more preferably less than about 2.0%, even more preferably less than about 1.0%, still more preferably less than about 0.5%, still even more preferably less than about 0.2% and most preferably less than about 0.08%.

5 The solvent may preferably be a non-aqueous solvent, more preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation, and most preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation and that has little or no dissolved oxygen or other gas(es) that is (are) prone to the formation of free-radicals upon irradiation. Volatile non-aqueous solvents
10 are particularly preferred, even more particularly preferred are non-aqueous solvents that are stabilizers, such as ethanol and acetone.

15 In certain embodiments of the present invention, the solvent may be a mixture of water and a non-aqueous solvent or solvents, such as ethanol and/or acetone. In such embodiments, the non-aqueous solvent(s) is preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation, and most preferably a non-aqueous solvent that is not prone to the formation of free-radicals upon irradiation and that has little or no dissolved oxygen or other gas(es) that is (are) prone to the formation
20 of free-radicals upon irradiation. Volatile non-aqueous solvents are particularly preferred, even more particularly preferred are non-aqueous solvents that are stabilizers, such as ethanol and acetone.

25 In a preferred embodiment, when the residual solvent is water, the residual solvent content of a biological material is reduced by dissolving or suspending the biological material in a non-aqueous solvent that is capable of dissolving water. Preferably, such a non-aqueous solvent is not prone to the formation of free-radicals upon irradiation and has little or no dissolved oxygen or other gas(es) that is (are) prone to the formation of free-radicals upon irradiation.

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intended use of the preparation of one or more digestive enzymes being irradiated, and can be determined empirically by one skilled in the art. An "unacceptable level" of damage would therefore be a level of damage that would preclude the safe and effective use of the preparation of one or more digestive enzymes being sterilized. The particular level of damage in a given preparation of one or more digestive enzymes may be determined using any of the methods and techniques known to one skilled in the art.

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The residual solvent content of a preparation of one or more digestive enzymes may be reduced by any of the methods and techniques known to those skilled in the art for reducing solvent from a preparation of one or more digestive enzymes without producing an unacceptable level of damage to the preparation. Such methods include, but are not limited to, evaporation, concentration, centrifugal concentration, vitrification, addition of solute, lyophilization (with or without the prior addition of ascorbate) and spray-drying.

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A particularly preferred method for reducing the residual solvent content of a preparation of one or more digestive enzymes is lyophilization, even more preferred is lyophilization following the addition of ascorbate.

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Another particularly preferred method for reducing the residual solvent content of a preparation of one or more digestive enzymes is vitrification, which may be accomplished by any of the methods and techniques known to those skilled in the art, including the addition of solute and or additional solutes, such as sucrose, to raise the eutectic point of the biological material, followed by a gradual application of reduced pressure to the biological material in order to remove the residual solvent, such as water. The resulting glassy material will then have a reduced residual solvent content.

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According to certain methods of the present invention, the preparation of one or more digestive enzymes to be sterilized may be immobilized upon a solid surface by any means known and available to one skilled in the art. For example, the preparation of one

kGy/hour, still even more preferably between about 0.5 kGy/hr and 1.5 kGy/hr and most preferably between about 0.5 kGy/hr and 1.0 kGy/hr.

According to another particularly preferred embodiment of the present invention, the rate of irradiation is at least about 3.0 kGy/hr., more preferably at least about 6 kGy/hr., even more preferably at least about 16 kGy/hr., and even more preferably at least about 30 kGy/hr and most preferably at least about 45 kGy/hr or greater.
5

According to the methods of the present invention, the preparation of one or more digestive enzymes to be sterilized is irradiated with the radiation for a time effective for the inactivation of one or more biological contaminants or pathogens of the preparation of one or more digestive enzymes. Combined with irradiation rate, the appropriate irradiation time results in the appropriate dose of irradiation being applied to the preparation of one or more digestive enzymes. Suitable irradiation times may vary depending upon the particular form and rate of radiation involved, the nature and characteristics of the particular preparation of one or more digestive enzymes being irradiated and/or the particular biological contaminants or pathogens being inactivated.
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15
Suitable irradiation times can be determined empirically by one skilled in the art.

According to the methods of the present invention, the preparation of one or more digestive enzymes to be sterilized is irradiated with radiation up to a total dose effective for the inactivation of one or more active biological contaminants or pathogens in the material, while not producing an unacceptable level of damage to that material. Suitable total doses of radiation may vary depending upon certain features of the methods of the present invention being employed, such as the nature and characteristics of the particular preparation being irradiated, the particular form of radiation involved and/or the particular active biological contaminant or pathogen being inactivated. Suitable total doses of radiation can be determined empirically by one skilled in the art. Preferably, the total dose of radiation is at least 25 kGy, more preferably at least 45 kGy, even more preferably at least 75 kGy, and still more preferably at least 100 kGy or greater, such as 150 kGy or 200 kGy or greater.
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Most preferably, the irradiation of the preparation of one or more digestive enzymes occurs at a temperature that protects the preparation from radiation. Suitable temperatures can be determined empirically by one skilled in the art.

In certain embodiments of the present invention, the temperature at which 5 irradiation is performed may be found to lie within a range, rather than at a specific point. Such a range for the preferred temperature for the irradiation of a particular preparation of one or more digestive enzymes may be determined empirically by one skilled in the art.

According to the methods of the present invention, the irradiation of the 10 preparation of one or more digestive enzymes may occur at any pressure which is not deleterious to the biological material being sterilized. According to one preferred embodiment, the preparation of one or more digestive enzymes is irradiated at elevated pressure. More preferably, the preparation of one or more digestive enzymes is irradiated at elevated pressure due to the application of sound waves, the use of a volatile, compression or other means known to those skilled in the art. While not wishing to be bound by any theory, the use of elevated pressure may enhance the effect of irradiation 15 on the biological contaminant(s) or pathogen(s) and/or enhance the protection afforded by one or more stabilizers, and therefore allow the use of a lower total dose of radiation. Suitable pressures can be determined empirically by one skilled in the art.

Generally, according to the methods of the present invention, the pH of the 20 preparation of one or more digestive enzymes undergoing sterilization is about 7. In some embodiments of the present invention, however, the preparation of one or more digestive enzymes may have a pH of less than 7, preferably less than or equal to 6, more preferably less than or equal to 5, even more preferably less than or equal to 4, and most preferably less than or equal to 3. In alternative embodiments of the present invention, the preparation of one or more digestive enzymes may have a pH of greater than 7, 25 preferably greater than or equal to 8, more preferably greater than or equal to 9, even more preferably greater than or equal to 10, and most preferably greater than or equal to

controlled reduction of pressure within a container (rigid or flexible) holding the preparation to be treated or by placing the preparation in a container of approximately equal volume.

It will be appreciated that the combination of one or more of the features described herein may be employed to further minimize undesirable effects upon the preparation of one or more digestive enzymes caused by irradiation, while maintaining adequate effectiveness of the irradiation process on the biological contaminant(s) or pathogen(s). For example, in addition to the use of a stabilizer, a particular preparation of one or more digestive enzymes may also be lyophilized, held at reduced temperature and kept under vacuum prior to irradiation to further minimize undesirable effects.

The sensitivity of a particular biological contaminant or pathogen to radiation is commonly calculated by determining the dose necessary to inactivate or kill all but 37% of the agent in a sample, which is known as the D_{37} value. The desirable components of a preparation of one or more digestive enzymes may also be considered to have a D_{37} value equal to the dose of radiation required to eliminate all but 37% of their desirable biological and physiological characteristics.

In accordance with certain preferred methods of the present invention, the sterilization of a preparation of one or more digestive enzymes is conducted under conditions that result in a decrease in the D_{37} value of the biological contaminant or pathogen without a concomitant decrease in the D_{37} value of the preparation of one or more digestive enzymes. In accordance with other preferred methods of the present invention, the sterilization of a preparation of one or more digestive enzymes is conducted under conditions that result in an increase in the D_{37} value of the preparation of one or more digestive enzymes. In accordance with the most preferred methods of the present invention, the sterilization of a preparation of one or more digestive enzymes is conducted under conditions that result in a decrease in the D_{37} value of the biological contaminant or pathogen and a concomitant increase in the D_{37} value of the preparation of one or more digestive enzymes.

In the absence of stabilizer, lyophilized trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 74% of control activity at the higher residual solvent content level, *i.e.* about 2.4% water, and recovery of 85% of control activity at the lower residual solvent content level, *i.e.*, about 1.8% water.

- 5 In the presence of stabilizer, trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 97% of control activity at higher residual solvent content levels, *i.e.* about 3.7% water, and recovery of 86% of control activity at lower residual solvent content levels, *i.e.* about 0.7% water.

The results of this experiment are shown graphically in Figures 1A-1B.

10

Example 2

In this experiment, trypsin was irradiated (45 kGy at 1.6 kGy/hr. and 4°C) in the presence of a stabilizer (sodium ascorbate 200 mM) as either a liquid or lyophilized preparation at varying pH levels.

15

Method

1 ml of 1 mg/ml (about 3000 IU/ml) trypsin aliquots in the presence of 35 mM phosphate buffer and 200 mM sodium ascorbate were made at varying pH levels between 5 and 8.5, inclusive. 400 µl of each solution was placed in 3 ml vials and then lyophilized and gamma-irradiated. The remaining portion of each solution was gamma-irradiated as a liquid. Lyophilized and liquid samples were assayed at the same time, under the following conditions: Assay conditions: 5 U/well trypsin (50 U/ml) + BATNA substrate (1 mg/ml) was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15

1 ml aliquots of trypsin alone or with 200 mM sodium ascorbate (1 mg/ml) were placed in 3 ml vials and frozen overnight at -70°C. Samples were prepared in quadruplicate and subjected to lyophilization, utilizing primary and secondary drying cycles (20 hours total).

5 All samples were resuspended in 1 ml water, and then diluted 1:10 for assay. Assay conditions: 50 units/ml trypsin per well + BATNA substrate starting at 3000 µg/ml was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 10 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

15 In the absence of stabilizer, lyophilized trypsin exposed to gamma-irradiation showed recovery of 63% of control activity. In the presence of stabilizer, lyophilized trypsin exposed to gamma-irradiation showed recovery of 88% of control activity. The results of this experiment are shown graphically in Figures 3A-3B.

Example 4

20 In this experiment, trypsin that had been lyophilized (0.7% moisture) was irradiated (45 kGy at 1.867 kGy/hr at 3.2°C) alone or in the presence of a stabilizer (sodium ascorbate 100 mM) at varying levels of residual solvent content.

Method

25 1 ml aliquots of trypsin alone or with 100 mM sodium ascorbate (10 mg/ml) were placed in 3 ml vials and frozen overnight at -70°C. Samples were prepared in

a combination of a primary drying cycle and a secondary drying cycle (65 hours, sample temp 40°C, shelf temp 40°C, 10 mT).

All samples were resuspended in 1 ml water, and then diluted 1:10 for assay. Assay conditions: 50 units/ml trypsin per well + BAPNA substrate starting at 3000 µg/ml was serially diluted 3-fold down a 96-well plate. The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

In the absence of stabilizer, trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 74% of control activity at the higher residual solvent content level, *i.e.* about 5.8% water, and recovery of 77% of control activity at the lower residual solvent content level, *i.e.*, about 5.4% water.

In the presence of stabilizer, trypsin exposed to 45 kGy total dose gamma-irradiation showed recovery of 97% of control activity at higher residual solvent content levels, *i.e.* about 2.8% water, and recovery of 90% of control activity at lower residual solvent content levels, *i.e.* about 1.1% water.

The results of this experiment are shown graphically in Figures 5A-5B.

Example 6

In this experiment, trypsin suspended in polypropylene glycol 400 was subjected to gamma irradiation at varying levels of residual solvent (water) content.

In this experiment, an aqueous solution of trypsin was subjected to gamma irradiation at varying concentrations of a stabilizer (sodium ascorbate, alone or in combination with 1.5mM uric acid).

Method

5 Trypsin samples (5 Units/sample) were prepared with varying concentrations of sodium ascorbate, alone or in combination with 1.5mM uric acid. Samples were irradiated to a total dose of 45 kGy at a rate of 1.9 kGy/hr and a temperature of 4°C.

10 Assay conditions: 5 U/well trypsin (50 U/ml) + 50 µl BAPNA substrate (1 mg/ml). The assay was set up in two 96-well plates and absorption read at both 405 and 620 nm at 5 and 20 minutes. The absorption at 630 nm (background) was subtracted from the value at 405 nm to obtain a corrected absorption value. The change in this value over time between 5 and 15 minutes of reaction time was plotted and Vmax and Km determined in Sigma Plot using the hyperbolic rectangular equation).

Results

15 The irradiated samples containing at least 20mM ascorbate retained varying levels of trypsin activity compared to an unirradiated control. Samples containing 125mM or more ascorbate retained about 75% of the trypsin activity of an unirradiated control. Similar results were observed with samples containing ascorbate in combination with uric acid. The results of this experiment are shown graphically in Figure 7.

20

Example 8

In this experiment, the protective effect of ascorbate (200 mM) and a combination of ascorbate (200 mM) and Gly-Gly (200 mM) on two different frozen enzyme preparations (a glycosidase and a sulfatase) was evaluated.

Samples were prepared in 2 ml glass vials, each containing 52.6 μ l of a glycosidase solution (5.7 mg/ml), and either no stabilizer or a stabilizer of interest, and sufficient water to make a total sample volume of 300 μ l. Samples were irradiated with gamma radiation (45 kGy total dose, dose rate and temperature of either 1.616 kGy/hr
5 and -21.5°C or 5.35 kGy/hr and -21.9°C) and then assayed for structural integrity.

Structural integrity was determined by reverse phase chromatography. 10 μ l of sample were diluted with 90 μ l solvent A and then injected onto an Aquapore RP-300 (c-
8) column (2.1 x 30 mm) mounted in an Applied Biosystems 130A Separation System
Microbore HPLC. Solvent A: 0.1% trifluoroacetic acid; solvent B: 70% acetonitrile, 30%
10 water, 0.085% trifluoroacetic acid.

Results

Enzyme samples irradiated to 45 kGy in the absence of a stabilizer showed broadened and reduced peaks. Much greater recovery of material, as evidenced by significantly less reduction in peak size compared to control (Figure 9), was obtained
15 from the irradiated samples containing ascorbate or a combination of ascorbate and Gly-Gly.

Example 10

In this experiment, lyophilized trypsin was irradiated (45 kGy total dose at 1.9
20 kGy/hr. at 4°C) in the presence of Tris buffer (pH 7.6) or phosphate buffer (pH 7.5).

Method

Aliquots of a 1000 IU/ml trypsin solution were placed in 3 ml vials and then lyophilized and gamma-irradiated. The remaining portion of each solution was gamma-irradiated as a liquid. Samples were assayed under the following conditions: Assay
25 conditions: 5 U/well trypsin (50 U/ml) + BATNA substrate (1 mg/ml) was serially

without stabilizer, 0.7%. Lyophilized samples were irradiated with gamma radiation (45 kGy total dose at 1.8 kGy/hr and 4°C) and then assayed for structural integrity.

Structural integrity was determined by SDS-PAGE. In an electrophoresis unit, 6 µg/lane of each sample was run at 120V on a 7.5%-15% acrylamide gradient gel with a 4.5% acrylamide stacker under non-reducing conditions.

Results

Lyophilized glycosidase samples irradiated to 45 kGy in the absence of a stabilizer showed significant recovery of intact enzyme with only some fragmentation. Fragmentation was reduced by the addition of a stabilizer.

Similarly, lyophilized sulfatase samples irradiated to 45 kGy in the absence of a stabilizer showed good recovery of intact enzyme, but with slightly more fragmentation. Fragmentation was again reduced by the addition of a stabilizer.

The results of this experiment are shown in Figure 10.

Example 12

In this experiment, lyophilized glycosidase preparations irradiated in the absence or presence of a stabilizer (200 mM sodium ascorbate or a combination of 200 mM ascorbate and 200 mM glycylglycine).

Methods

Samples were prepared in glass vials, each containing 300 µg of a lyophilized glycosidase and either no stabilizer or a stabilizer of interest. Samples were irradiated with gamma radiation to varying total doses (10 kGy, 30 kGy and 50 kGy total dose, at a

WHAT IS CLAIMED IS:

1. A method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation, said method comprising irradiating said preparation of one or more digestive enzymes with radiation for a time effective to sterilize said preparation of one or more digestive enzymes at a rate effective to sterilize said preparation of one or more digestive enzymes and to protect said preparation of one or more digestive enzymes from said radiation.

2. A method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation, said method comprising:

10 (i) adding to said preparation of one or more digestive enzymes at least one stabilizer in an amount effective to protect said preparation of one or more digestive enzymes from said radiation; and

15 (ii) irradiating said preparation of one or more digestive enzymes with a suitable radiation at an effective rate for a time effective to sterilize said preparation of one or more digestive enzymes.

3. A method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation, said method comprising:

20 (i) reducing the residual solvent content of said preparation of one or more digestive enzymes to a level effective to protect said preparation of one or more digestive enzymes from said radiation; and

(ii) irradiating said preparation of one or more digestive enzymes with a suitable radiation at an effective rate for a time effective to sterilize said preparation of one or more digestive enzymes.

4. A method for sterilizing a preparation of one or more digestive enzymes that is sensitive to radiation, said method comprising:

(a) reducing the residual solvent content of said preparation of one or more digestive enzymes,

(b) reducing the temperature of said preparation of one or more digestive enzymes, and

5 (c) adding at least one stabilizer to said preparation of one or more digestive enzymes; and

(ii) irradiating said preparation of one or more digestive enzymes with a suitable radiation at an effective rate for a time effective to sterilize said preparation of one or more digestive enzymes, wherein said at least two stabilizing processes are together effective to protect said preparation of one or more digestive enzymes from said radiation and further wherein said at least two stabilizing processes may be performed in any order.

7. The method according to claim 3, 5 or 6, wherein said solvent is water.

8. The method according to claim 7, wherein said residual water content is reduced by the addition of an organic solvent.

9. The method according to claim 3, 5 or 6, wherein said solvent is an organic solvent.

10. The method according to claim 3, 5 or 6, wherein said preparation of one or more digestive enzymes is suspended in an organic solvent following reduction of said residual solvent content.

11. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said effective rate is not more than about 3.0 kGy/hour.

12. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said effective rate is not more than about 2.0 kGy/hr.

25 13. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said effective rate is not more than about 1.0 kGy/hr.

25. The method according to claim 3, 5 or 6, wherein said residual solvent content is less than about 15%.

26. The method according to claim 3, 5 or 6, wherein said residual solvent content is less than about 10%.

5 27. The method according to claim 3, 5 or 6, wherein said residual solvent content is less than about 3%.

28. The method according to claim 3, 5 or 6, wherein said residual solvent content is less than about 2%.

10 29. The method according to claim 3, 5 or 6, wherein said residual solvent content is less than about 1%.

30. The method according to claim 3, 5 or 6, wherein said residual solvent content is less than about 0.5%.

31. The method according to claim 3, 5 or 6, wherein said residual solvent content is less than about 0.08%.

15 32. The method according to claim 1, 2, 3, 4, 5 or 6, wherein at least one sensitizer is added to said preparation of one or more digestive enzymes prior to said step of irradiating said preparation of one or more digestive enzymes.

20 33. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said preparation of one or more digestive enzymes contains at least one biological contaminant or pathogen selected from the group consisting of viruses, bacteria, yeasts, molds, fungi, prions or similar agents responsible, alone or in combination, for TSEs and single or multicellular parasites.

34. The method according to claim 2, 5 or 6, wherein said at least one stabilizer is an antioxidant.

25 35. The method according to claim 2, 5 or 6, wherein said at least one stabilizer is a free radical scavenger.

44. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is corpuscular radiation or electromagnetic radiation, or a mixture thereof.

45. The method according to claim 44, wherein said electromagnetic radiation is selected from the group consisting of radio waves, microwaves, visible and invisible light, ultraviolet light, x-ray radiation, gamma radiation and combinations thereof.

5 46. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is gamma radiation.

47. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is E-beam radiation.

10 48. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is visible light.

49. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is ultraviolet light.

15 50. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is x-ray radiation.

51. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is polychromatic visible light.

52. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is infrared.

20 53. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said radiation is a combination of one or more wavelengths of visible and ultraviolet light.

54. The method according to claim 1, 2, 3, 5 or 6, wherein said irradiation is conducted at ambient temperature.

25 55. The method according to claim 1, 2, 3, 4, 5 or 6, wherein said irradiation is conducted at a temperature below ambient temperature.

67. The composition of claim 60, wherein said residual solvent content is less than about 0.08%.

68. The composition of claim 59 or 60, wherein said preparation of one or more digestive enzymes is glassy or vitrified.

5 69. The composition of claim 59 or 60, wherein said preparation of one or more digestive enzymes contains at least one enzyme selected from the group consisting of trypsin, glycosidases and sulfatases.

70. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 0.5%.

10 71. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 1%.

72. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 5%.

15 73. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 10%.

74. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 15%.

75. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 20%.

20 76. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 25%.

77. The composition of claim 60, wherein the total protein concentration of said preparation of one or more digestive enzymes is at least about 50%.

25 78. A method of treating a digestive enzyme deficiency in a mammal comprising administering to a mammal in need thereof an effective amount of a preparation of one or more digestive enzymes which has been sterilized according to the method according to claim 1, 2, 3, 4, 5 or 6.

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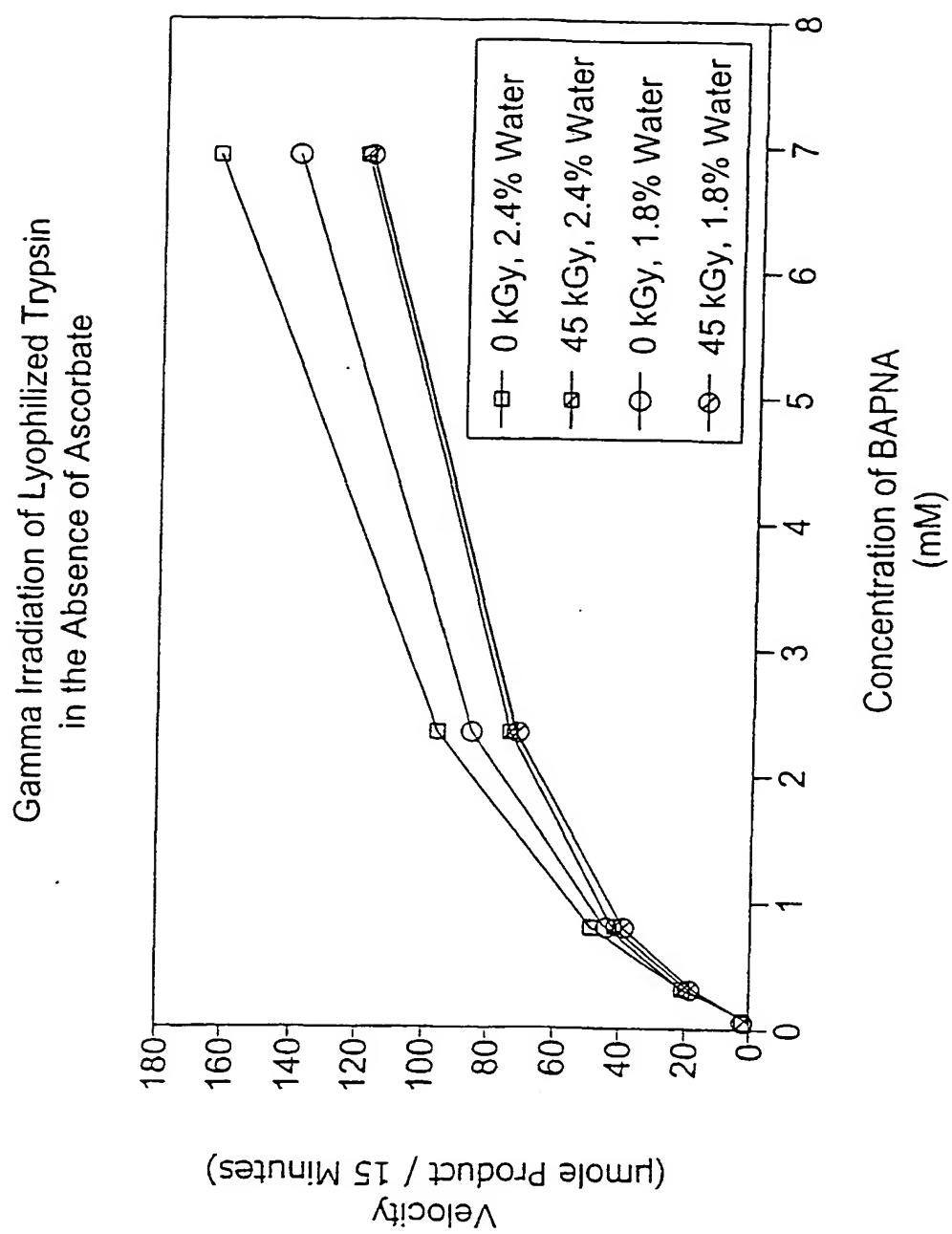


FIG. 1A

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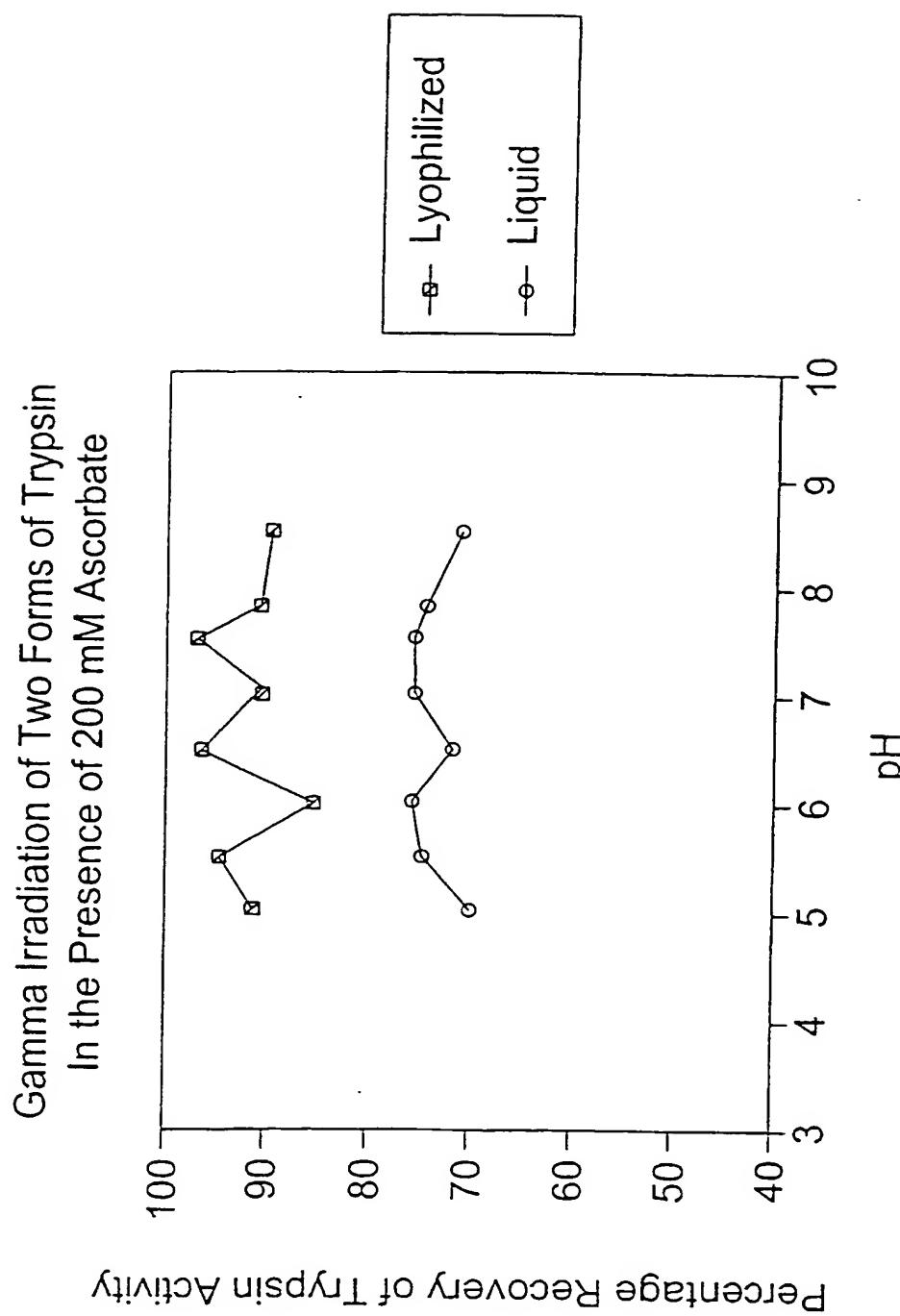


FIG. 2

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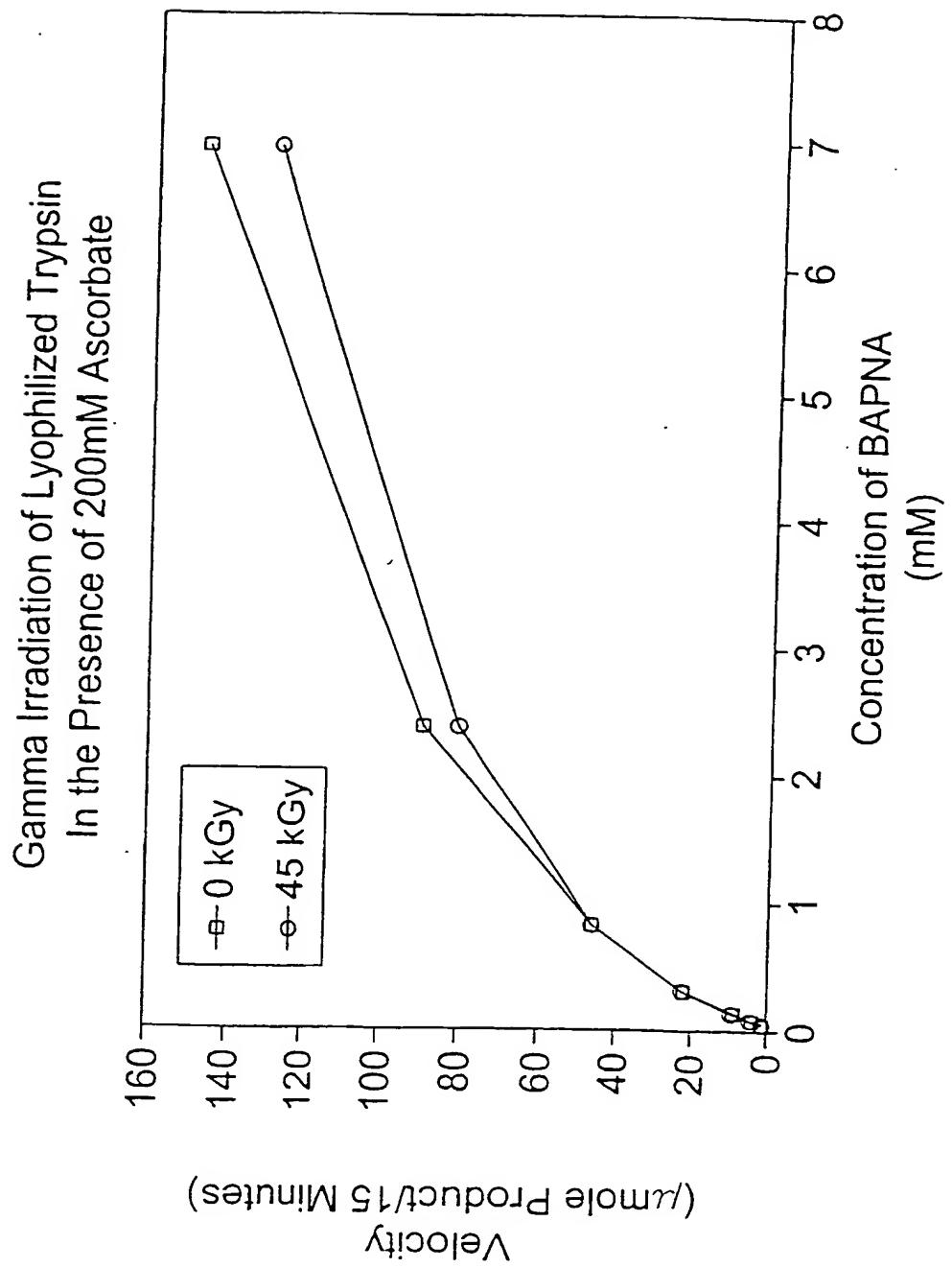


FIG. 3B

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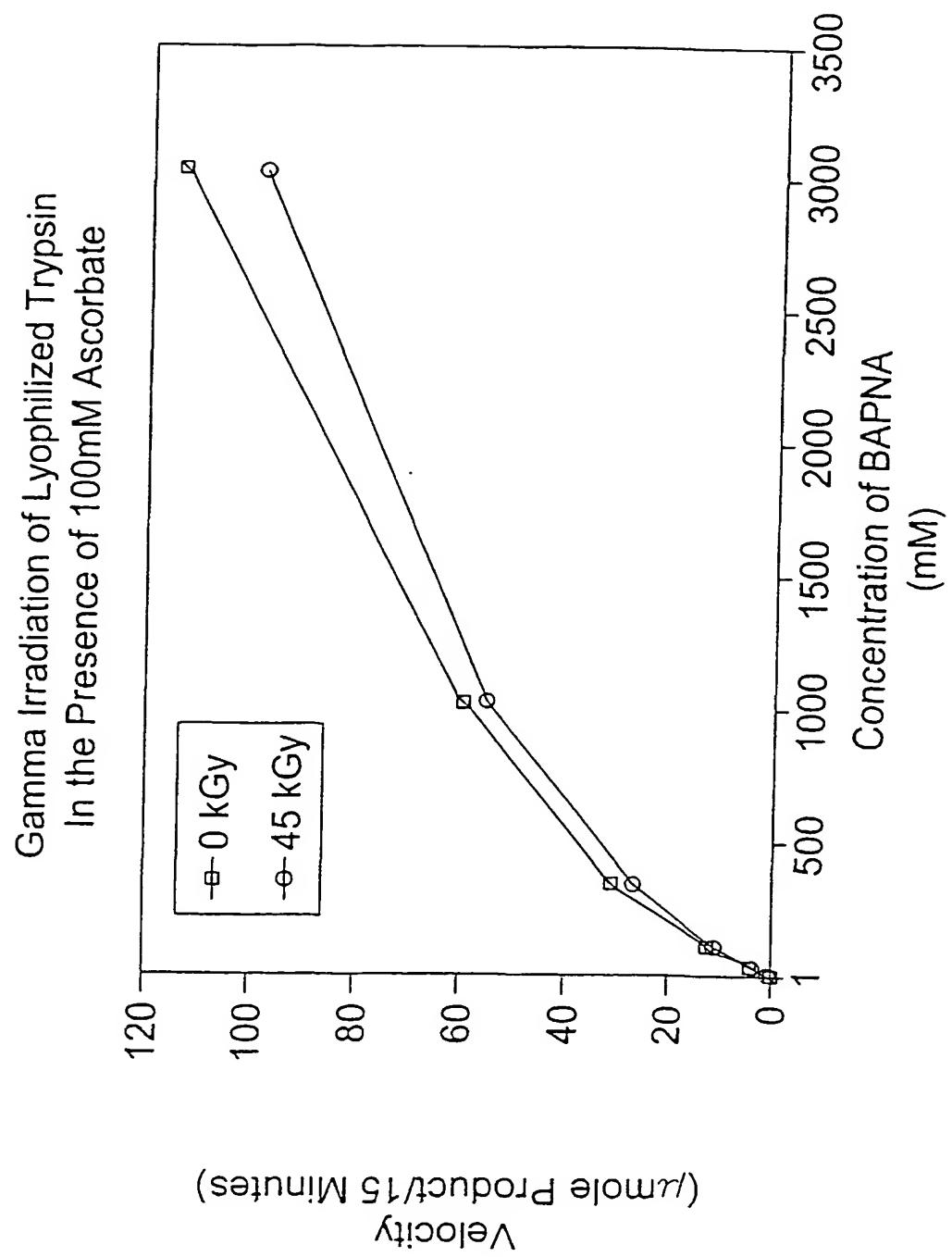


FIG. 4B

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Gamma Irradiation of Lyophilized Trypsin
In the Presence of 100 mM Ascorbate

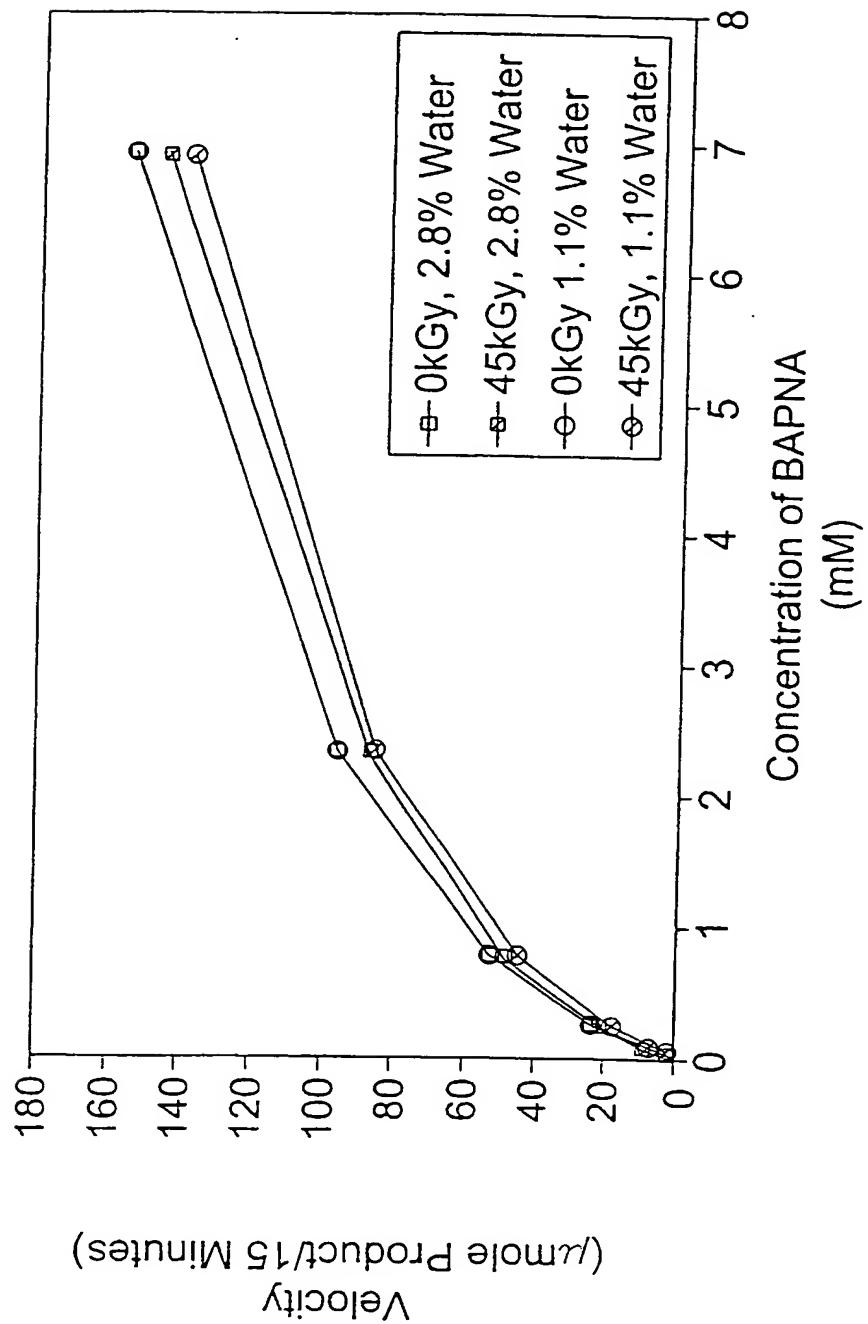
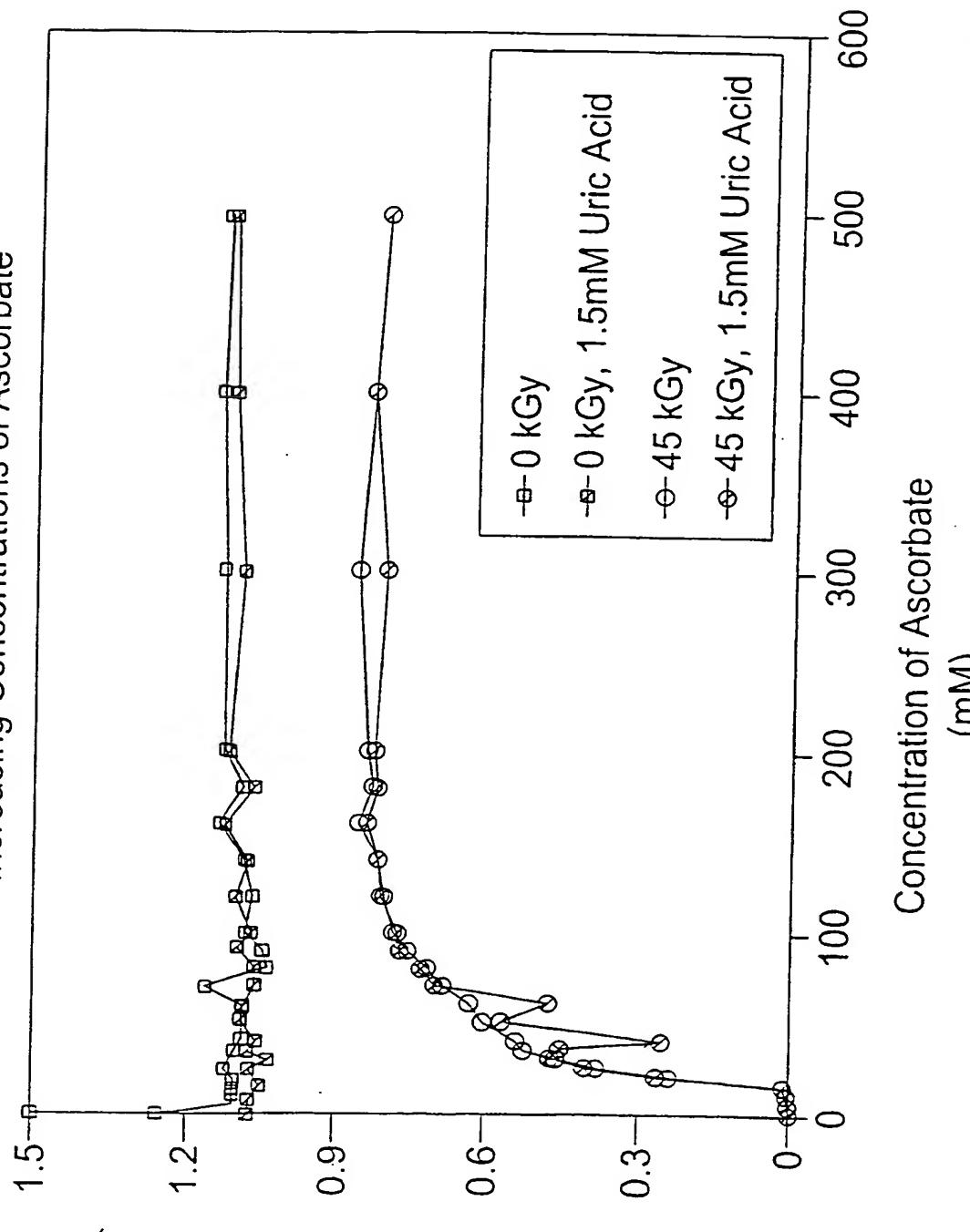


FIG. 5B

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Gamma Irradiation of Trypsin In the Presence of
Increasing Concentrations of Ascorbate



Trypsin Activity
(Absorbance at 405 nm Minus Absorbance at
620 nm, 20 Minutes Minus 2 Minutes)
620nm, 20 Minutes Minus 2 Minutes)

FIG. 7

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SDS-PAGE for a Sulfatse

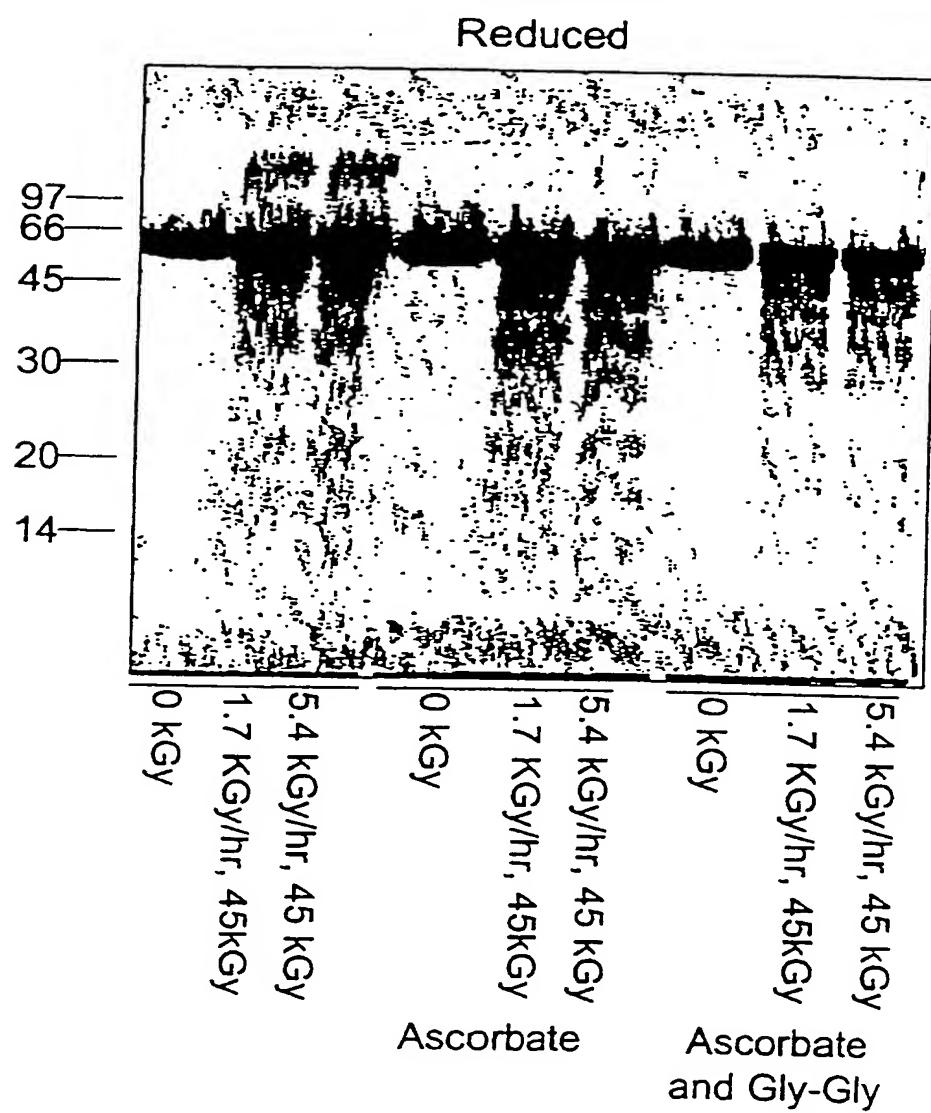


FIG. 8B

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Gamma Irradiation of a Lyophilized Glycosidase and Sulfatase In the Absence and Presence of 100mM Ascorbate

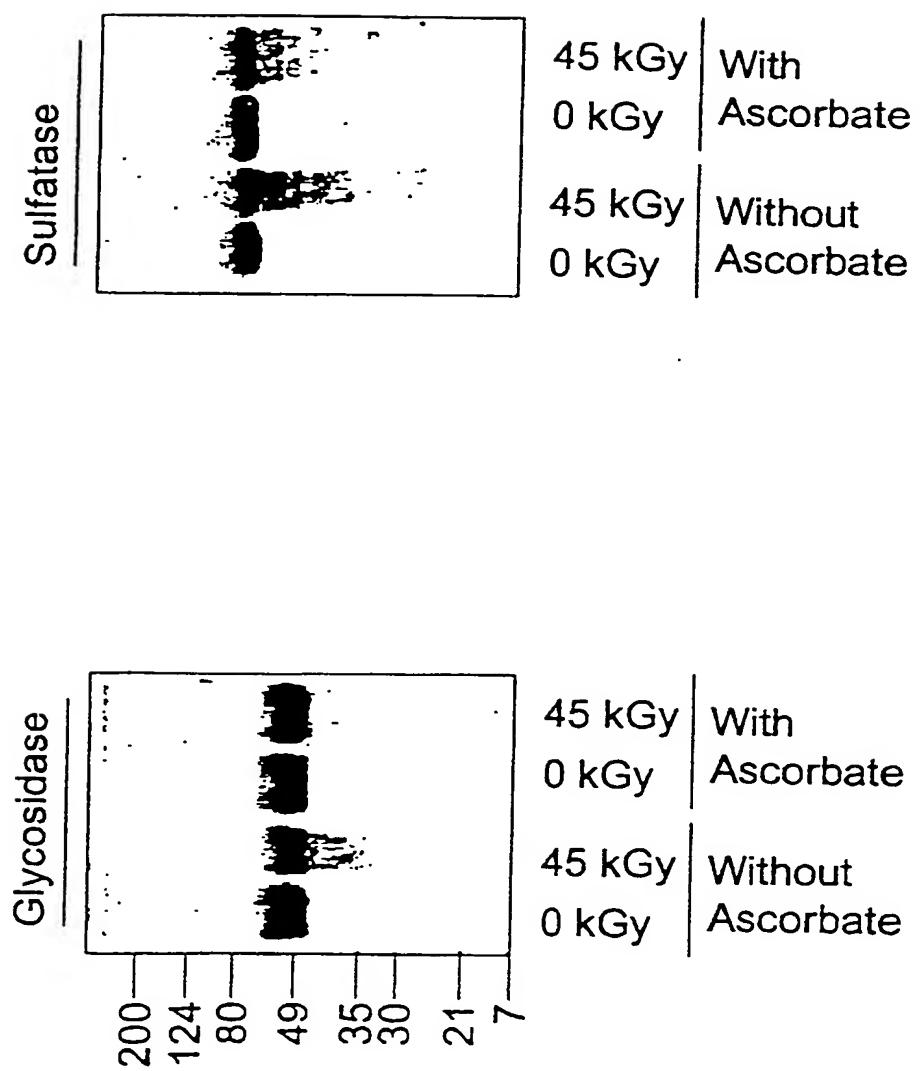


FIG. 10

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Gamma Irradiation of a Lyophilized Glycosidase
In the Presence of 200 mM Ascorbate

Reduced & Non-Reduced, 10%

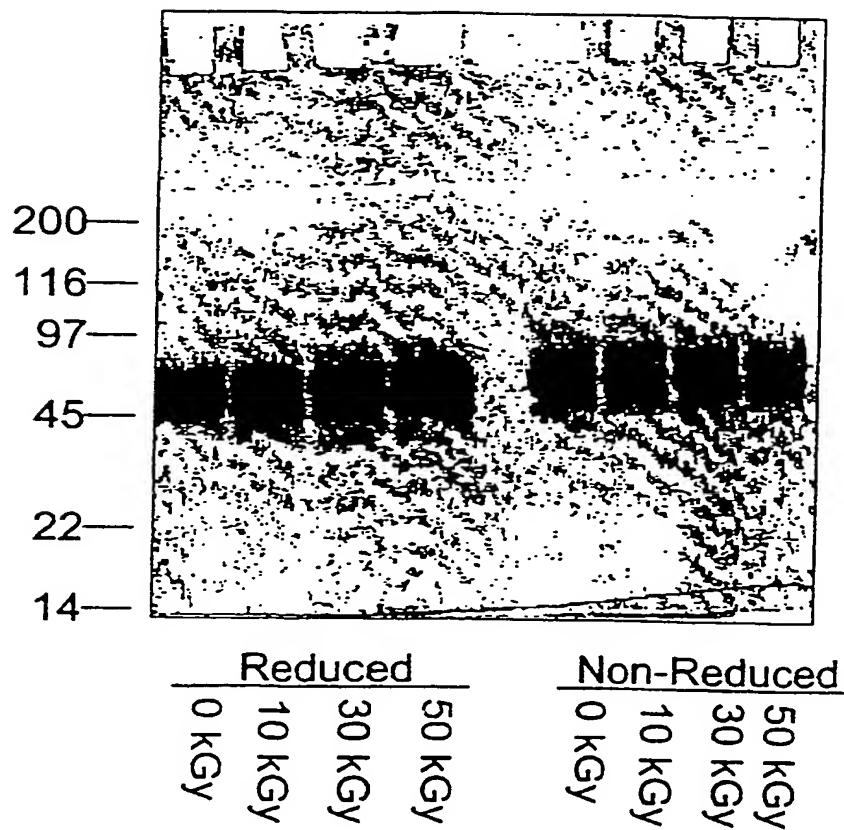


FIG. 11B

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Gamma Irradiation of Lyophilized Trypsin
in the Absence of Ascorbate

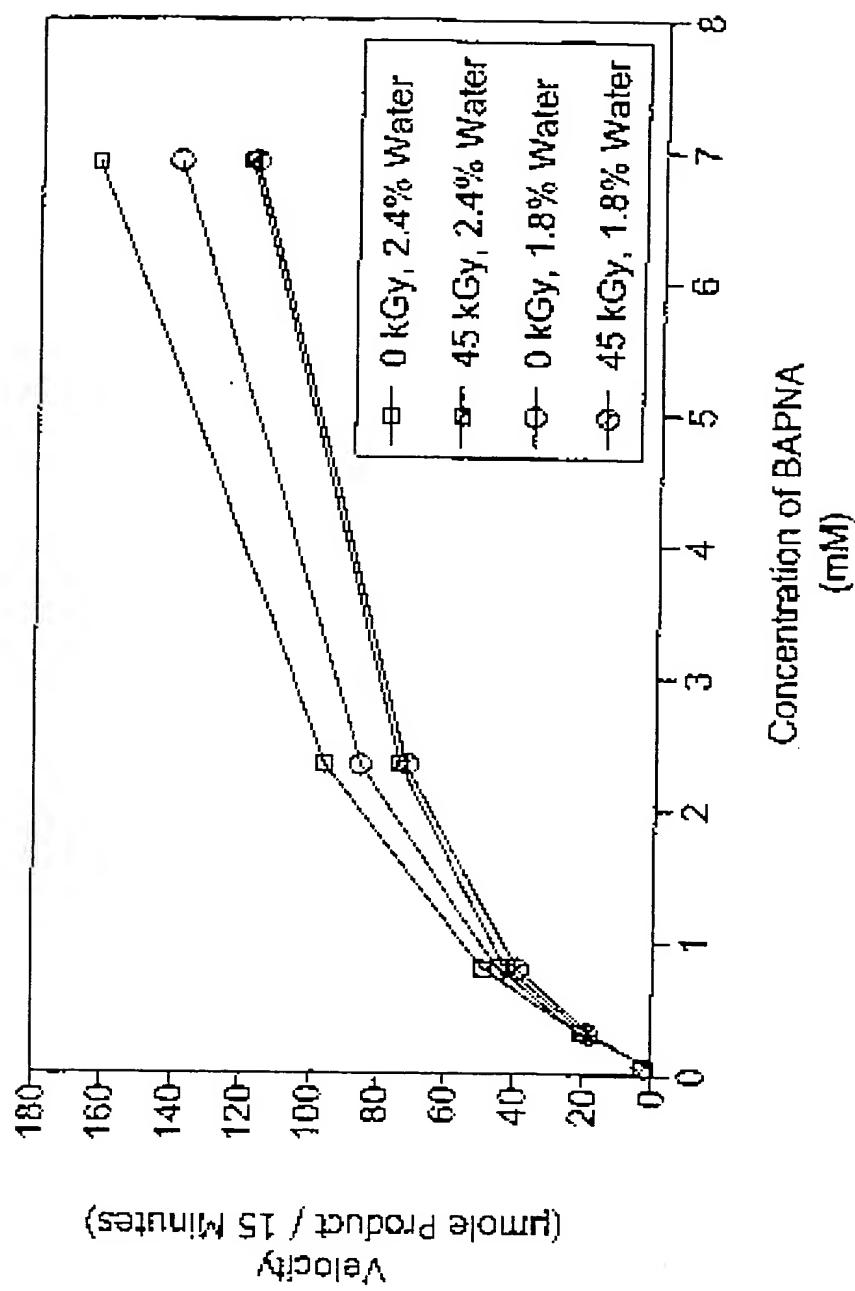


FIG. 1A

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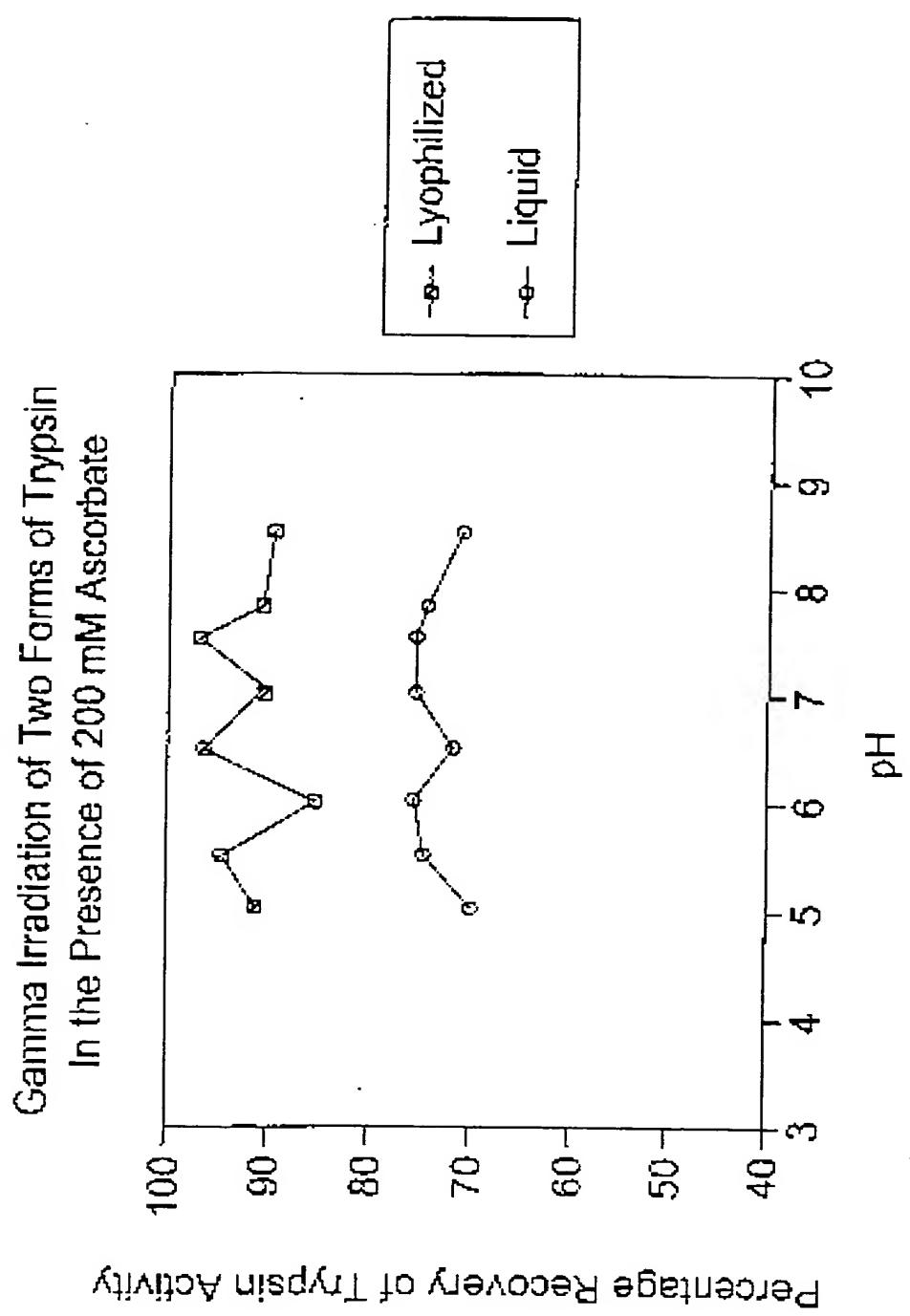


FIG. 2

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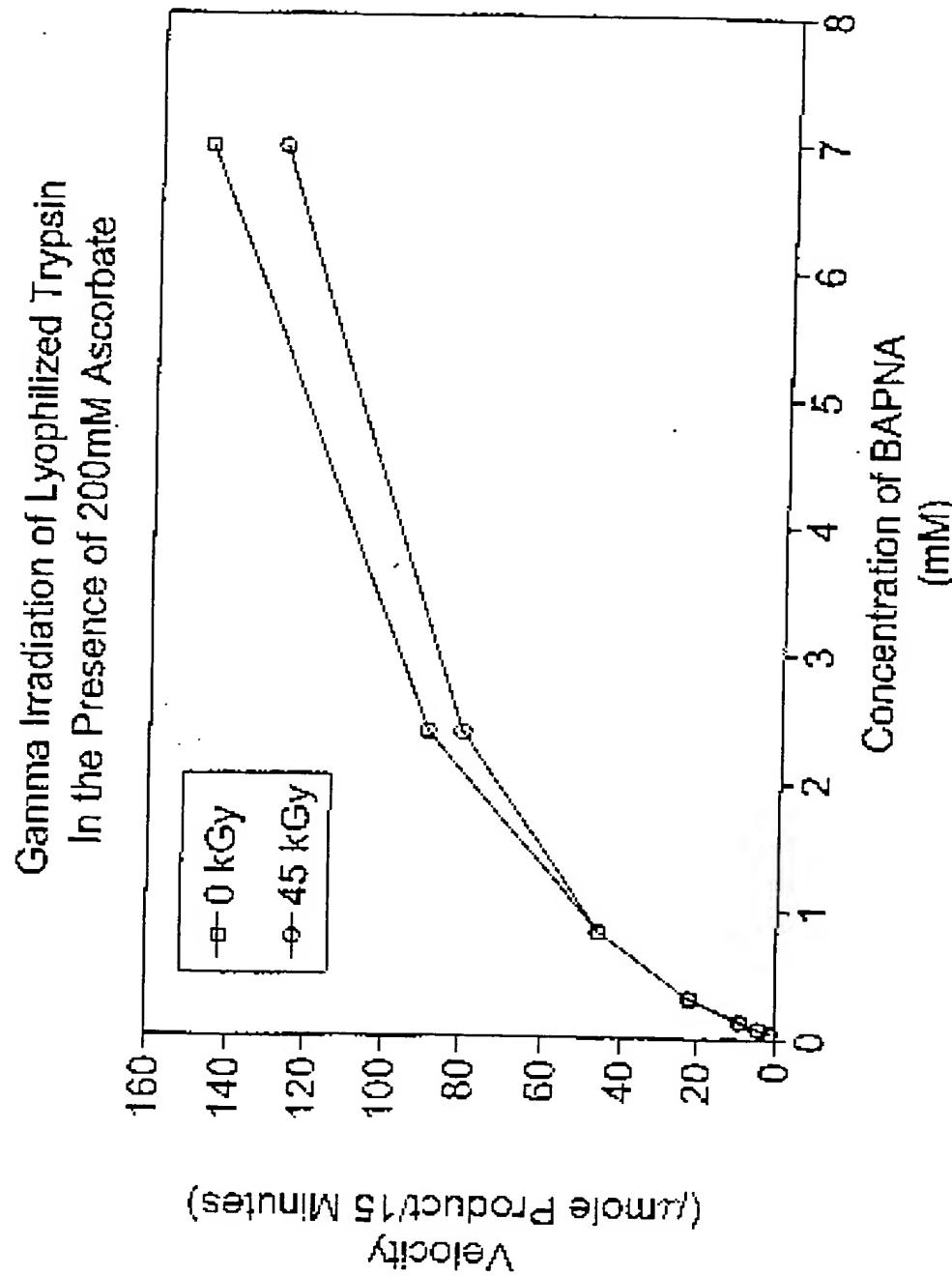


FIG. 3B

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Gamma Irradiation of Lyophilized Trypsin
In the Presence of 100mM Ascorbate

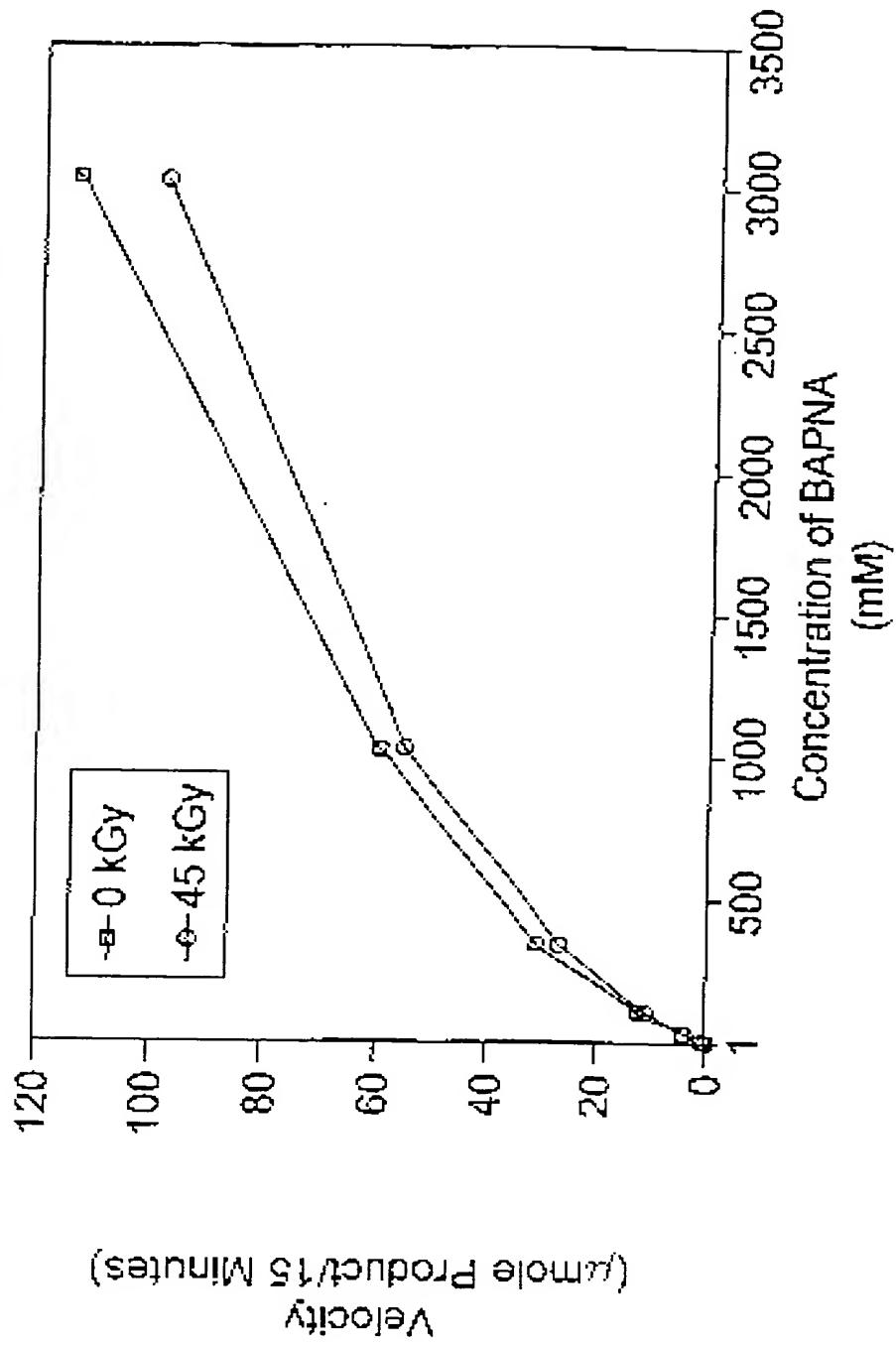


FIG. 4B

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Gamma Irradiation of Lyophilized Trypsin
In the Presence of 100 mM Ascorbate

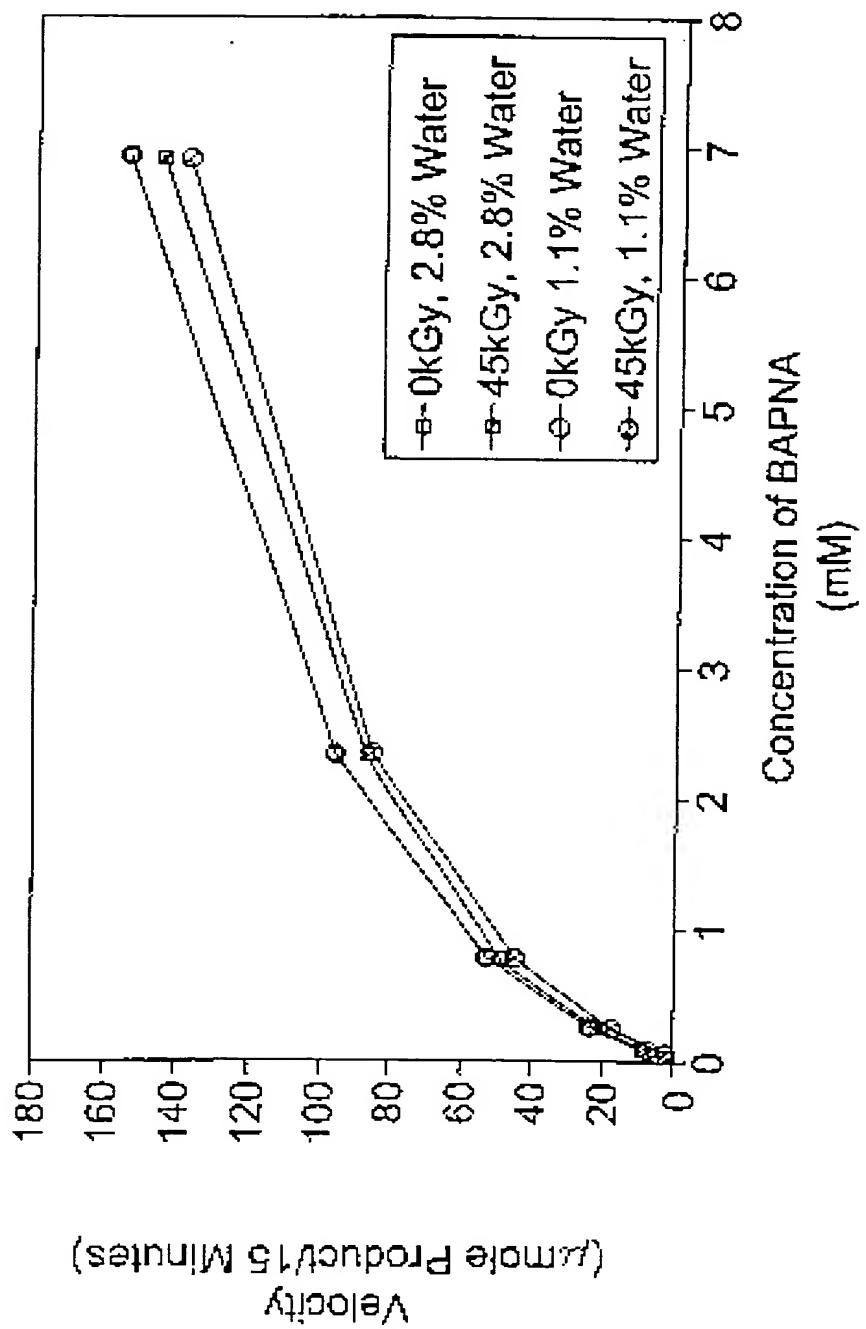


FIG. 5B

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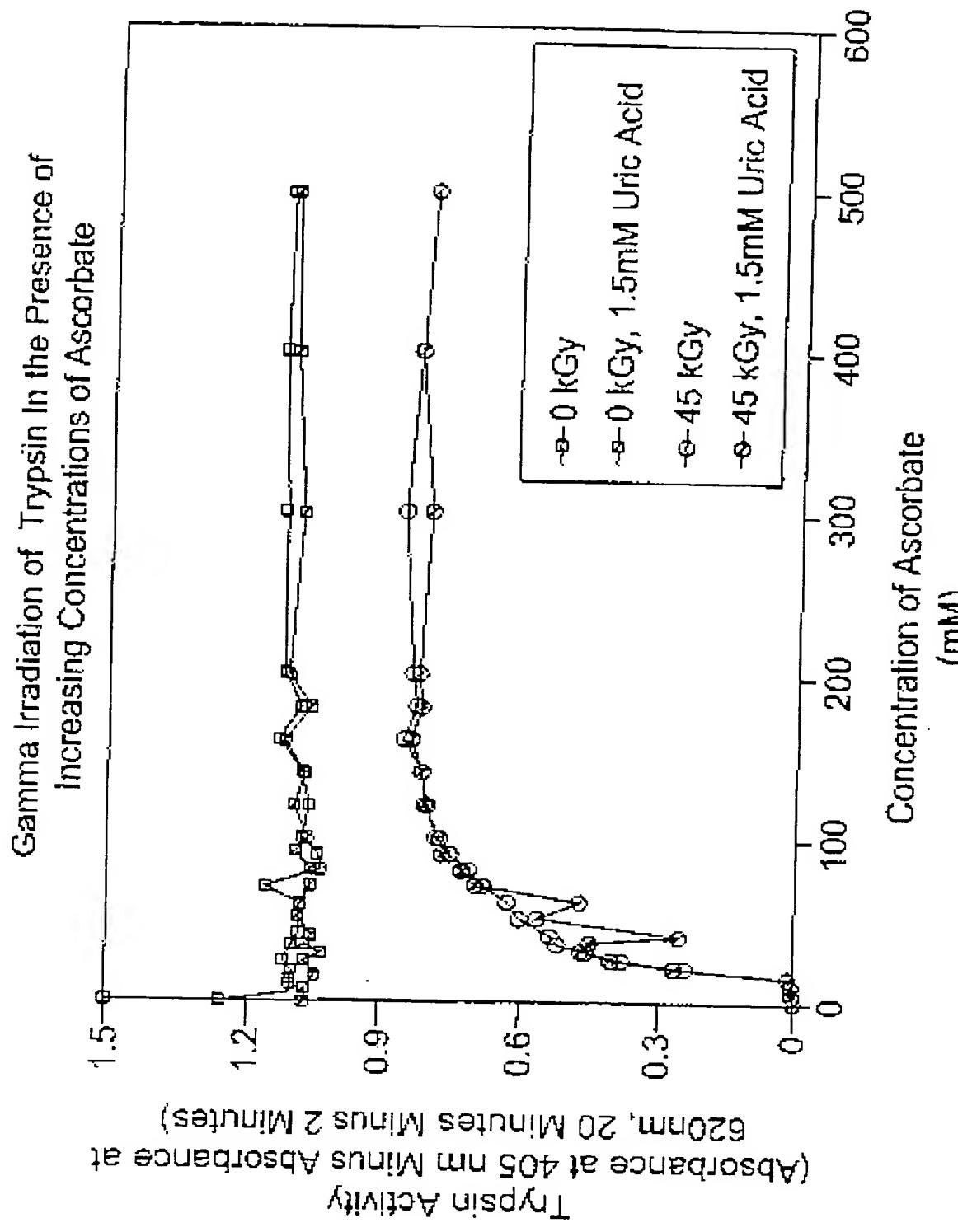


FIG. 7

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SDS-PAGE for a Sulfatase

Reduced

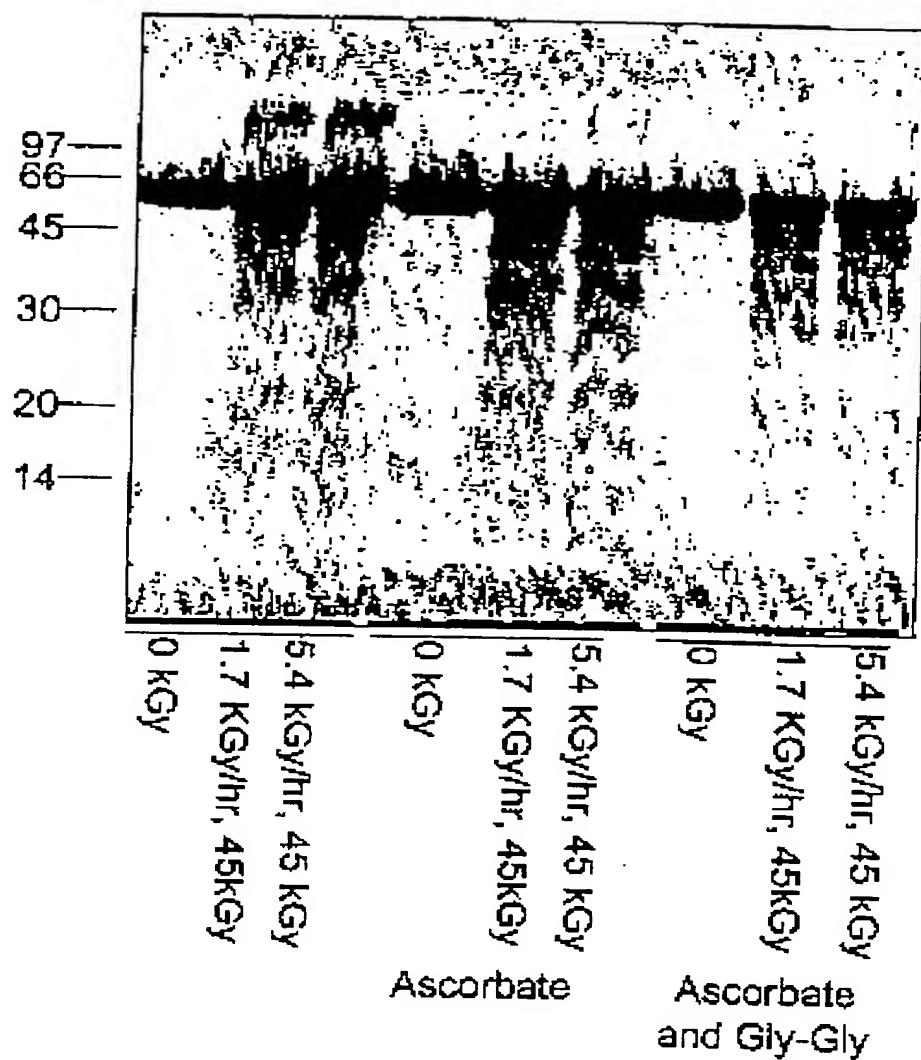


FIG. 8B

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Gamma Irradiation of a Lyophilized Glycosidase
and Sulfatase In the Absence and Presence of
100mM Ascorbate

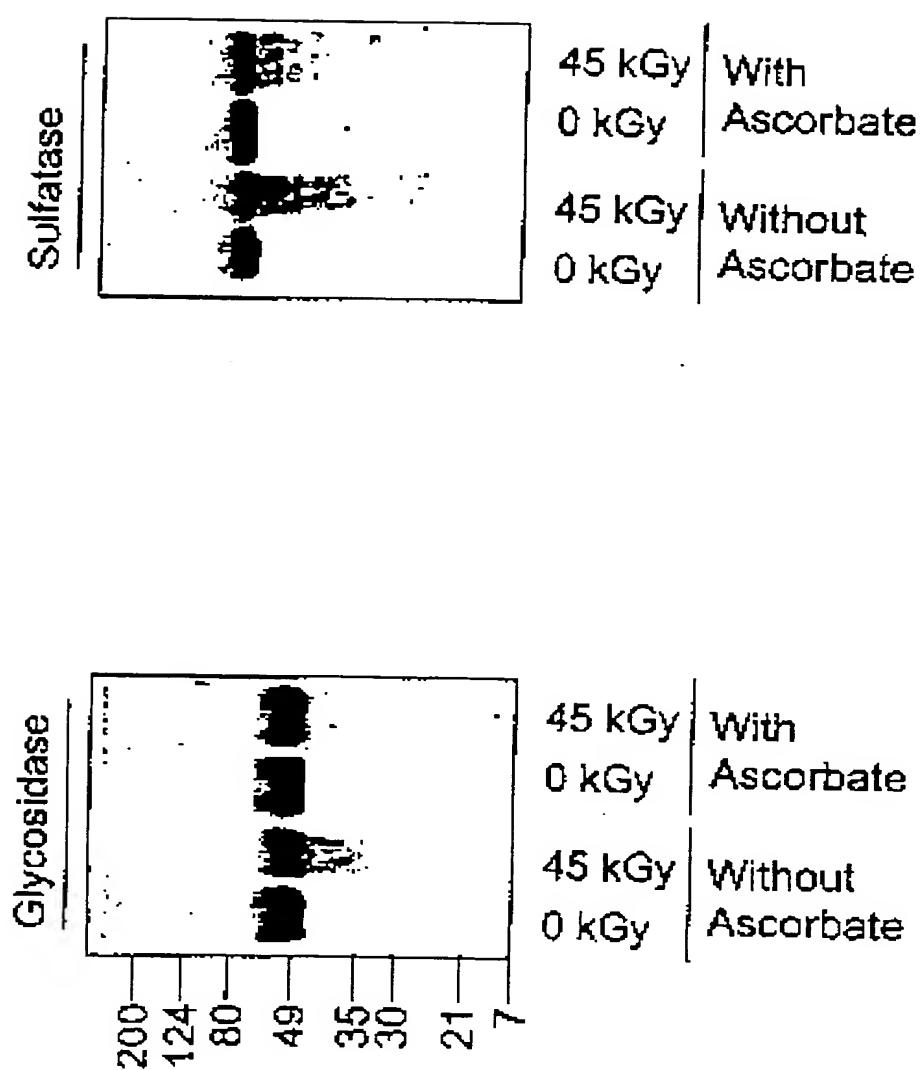


FIG. 10

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**Gamma Irradiation of a Lyophilized Glycosidase
In the Presence of 200 mM Ascorbate**

Reduced & Non-Reduced, 10%

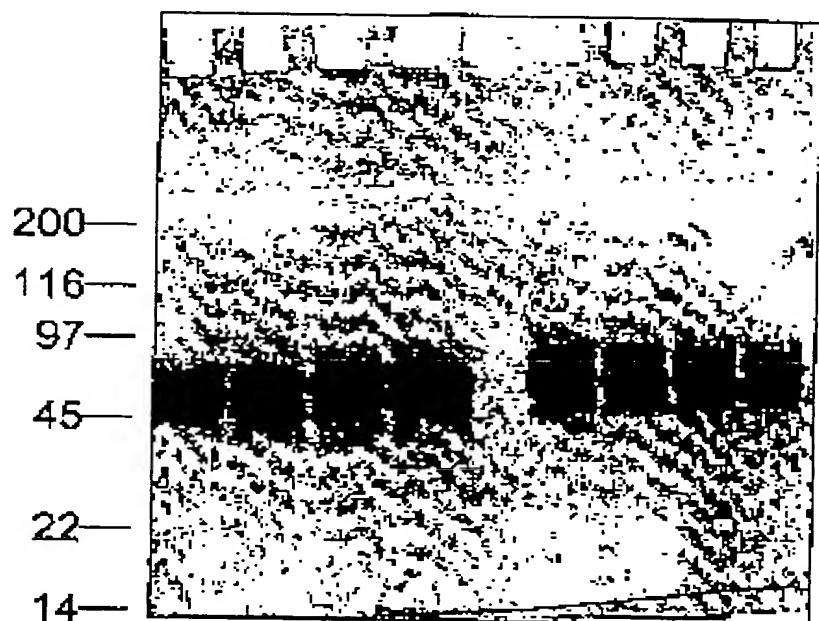


FIG. 11B

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